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POTENTIAL OF NOVEL PROBIOTICS ISOLATED FROM FISH GUT MICROBIOTA FOR IMPROVING PLANT FEEDSTUFFS UTILIZATION AND GUT HEALTH IN WHITE SEABREAM (*Diplodus sargus*) JUVENILES

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Dissertação de Mestrado apresentada à
Faculdade de Ciências da Universidade do Porto em
Recursos Biológicos Aquáticos

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Departamento de Biologia

2016

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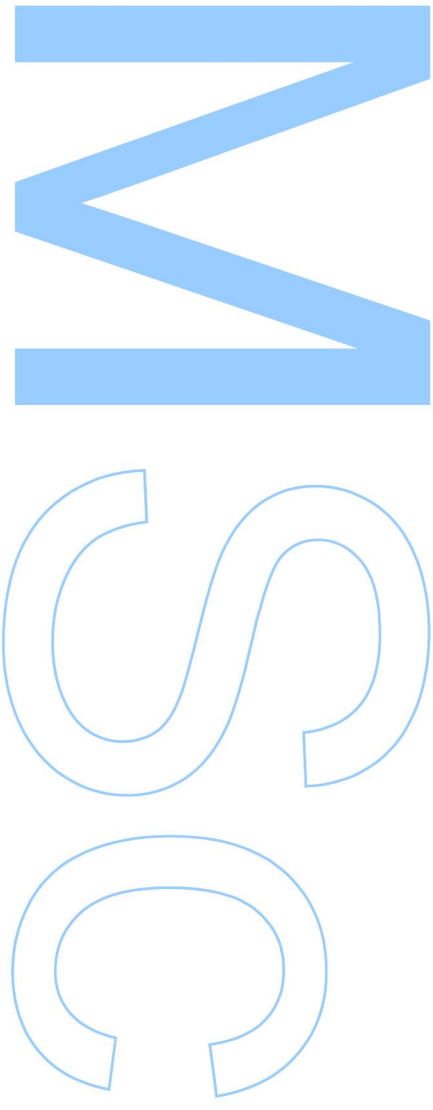
Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____



"*The sea is everything. It covers seven tenths of the terrestrial globe. Its breath is pure and healthy. It is an immense desert, where man is never lonely, for he feels life stirring on all sides. The sea is only the embodiment of a supernatural and wonderful existence. It is nothing but love and emotion; it is the Living Infinite.*"

Julio Verne, Twenty Thousand Leagues Under the Sea

Acknowledgements

I would like to start by expressing my gratitude to Prof. Dr. Aires Oliva-Teles, for the opportunity given by accepting me as an MSC student in Nutrimu Lab.

Again, I would like to express my gratitude to Paula Enes for sharing the huge amount of scientific knowledge, the capabilities and for all the guidance that was essential to complete this work.

Huge thanks to Ana Couto for sharing the scientific knowledge and capabilities that I needed to finish this work.

A very special thanks to Cláudia Serra, although not being my supervisor, for sharing the scientific knowledge and for the total availability to help in this work.

A very special thanks to all the co-workers in the group for all the help given every time I needed it.

Very special thanks for my friends for the support in all the moments throughout the years.

Huge thanks to all my family for the unconditional support and motivation that I needed to complete my education. I owe all that I accomplished to them.

Abstract

In the last decades, the aquaculture industry has been relying in fishmeal (FM) as the main protein source in aquafeeds. However, due to FM rising prices and limited supplies, the aquaculture sector has reduced their FM reliance by using alternative protein sources. Plant feedstuffs (PF) are the main alternative to FM due to their reasonable price and high availability. On the down side, PF have low protein content, several essential amino acids inadequacies, low palatability, and a wide range of anti-nutrients including non-starch polysaccharides (NSPs). In fish, enzymes needed to hydrolyze NSPs are rare or even absent, making them indigestible and unavailable as energy source. NSPs may also interfere with fish performance and gut health. Probiotic (PRO) bacteria capable of producing enzymes that hydrolyses NSPs can be used to allow the host to obtain energy from otherwise indigestible dietary constituents and therefore, reduce their harmful effects on the gut.

The present work is a follow-up of a research project that isolated gut spore-forming bacteria producing high levels of NSPs hydrolysing enzymes and complying with the minimum safety requirements to be eligible as PRO by the European Food Safety Authority. Thus, this work aimed at evaluating the most effective PRO to enhance the use of diets with a high PF content in white seabream (*Diplodus sargus*) juveniles, without compromising fish health and welfare. PRO efficiency was evaluated through assessing their effects on growth performance; feed utilization efficiency; the modulation of gut microbiota; digestive enzymes activities; and gut integrity. For that purpose, a control (CTR) diet was formulated to contain 48% of protein and 18% of lipid. A ratio of 20:80 of protein from FM:PF, respectively was used. Two other diets were formulated identical to the CTR diet but including 2 lyophilised pure spores preparations of *Bacillus* sp., FI99 and FI162 diets, respectively (2 x 10^9 spores g^{-1} feed). The trial lasted 65 days and during that period white seabream (54 g) were fed by hand, until apparent visual satiation. Growth performance was similar among experimental treatments. Feed, N and lipid intake were also not affected by PRO incorporation. Although not statistically significant, feed efficiency and protein efficiency ratio of fish fed the FI99 and FI162 diets were slightly higher compared to the CTR diet. In both autochthonous and allochthonous gut microbiota the average number of operational taxonomic units, microbial richness, diversity, and similarity were not affected by dietary PRO supplementation. α-Amylase, lipase, total alkaline protease and trypsin activities were similar among groups. The distal intestine of white seabream showed no signs of inflammation. Gut

morphology was unaffected by dietary PRO incorporation. Overall, dietary PRO supplementation failed in modulating both autochthonous and allochthonous gut microbiota, and thus based on the parameters assessed and considering cost-benefit, does not seem worthy of including these PRO in white seabream juveniles PF-based diets.

Keywords: Gut health; Microbiota; Non-starch polysaccharides; Plant feedstuffs; Probiotics; Sustainable aquafeeds

Resumo

Nas últimas décadas, a indústria da aquacultura tem estado dependente da farinha de peixe (FP) como principal fonte proteica das dietas. Contudo, devido ao seu elevado preço e disponibilidade limitada, o sector da aquacultura reduziu o consumo de FP através do uso de fontes proteicas alternativas. As matérias-primas vegetais (MPV) são a principal fonte alternativa à FP com um preço razoável e elevada disponibilidade. Como desvantagens, possuem um baixo teor proteico, deficiências em aminoácidos essenciais, baixa palatabilidade e vários anti-nutrientes dentre os quais os polissacarídeos não-amiláceos (NSPs). Nos peixes, as enzimas necessárias para hidrolisar os NSPs estão ausentes, tornando-os indigeríveis e indisponíveis como fonte energética. Os NSPs podem igualmente interferir na performance do peixe e na saúde intestinal. Bactérias probióticas (PRO) capazes de produzir enzimas que hidrolisem os NSPs poderão ser usadas para permitir ao hospedeiro obter energia de moléculas indigeríveis bem como reduzir os seus efeitos adversos no intestino. Este trabalho surge na continuidade de um projeto que isolou bactérias formadoras de esporos com capacidade de produzir enzimas que hidrolisem os NSPs. Estas bactérias obedecem aos requerimentos mínimos da Autoridade Europeia para a Segurança dos Alimentos para poderem ser utilizadas como PRO. Assim, o objetivo deste trabalho é avaliar a eficiência dos PRO em melhorar a utilização de dietas com elevados teores de MPV em juvenis de sargo (*Diplodus sargus*), sem comprometer a saúde e bem-estar do peixe. A eficiência dos PRO foi avaliada através dos seus efeitos no crescimento; eficiência alimentar; modulação do microbiota; atividade das enzimas digestivas; e integridade do intestino. Para esse fim, foi formulada uma dieta controlo (CTR) contendo 48% de proteína e 18% de lípidos. Foi usado um rácio proteico de 20:80 de FP:MPV, respetivamente. Outras duas dietas foram formuladas de maneira

idêntica à dieta CTR mas incluindo duas preparações de esporos puros liofilizados de *Bacillus* sp., FI99 e FI162, respetivamente (2 x 10^9 esporos g^{-1} alimento). O ensaio durou 65 dias e, durante esse período os sargos (54 g) foram alimentados à mão, até à saciedade aparente. O crescimento foi idêntico entre os tratamentos experimentais. O alimento, N e lípidos ingeridos também não foram afetados pela incorporação dos PRO nas dietas. Embora não estatisticamente significativo, a eficiência alimentar e o rácio de eficiência proteica dos peixes alimentados com as dietas FI99 e FI162 foram superiores comparativamente à dieta CTR. No microbiota autóctone e alóctone o número de unidades taxonómicas operacionais, a riqueza, a diversidade e similaridade não foram afetados pela suplementação dos PRO na dieta. A atividade da α-amílase, lípase, protéases totais alcalinas e tripsina foi similar entre os grupos. O intestino distal não apresentou sinais de inflamação. A morfologia do intestino não foi afetada pela incorporação dos PRO nas dietas. No geral, a suplementação das dietas com PRO não modulou o microbiota intestinal. Assim, e com base nos parâmetros avaliados e considerando o binómio custo-benefício, não parece vantajosa a incorporação destas estirpes em dietas à base de MPV para juvenis de sargo.

Palavra-chave: Saúde intestinal; Microbiota; Polissacarídeos não-amiláceos; Matérias-primas vegetais; Probióticos; Rações sustentáveis

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Abbreviations

CFU - Colony forming units

DI - Distal intestine

DGGE - Denaturing Gradient Gel Electrophoresis

FE - Feed efficiency

FM - Fishmeal

FO - Fish oil

GI - Gastrointestinal

IEL - Intraephitelial leukocytes

LAB - Lactic Acid Bacteria

LC-PUFA - Long-chain polyunsaturated fatty acids

NSPs - Non-starch polysaccharides

PCR - Polymerase chain reaction

PF - Plant feedstuffs

PRO - Probiotic

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1. Introduction

1.1. Aquaculture production

In the last five decades, global fish production has grown steadily at an average annual rate of 3.2% (Fig. 1), being the highest growth among animal production sectors (FAO, 2016). In global capture fishery, the production in 2014 was 93.4 million tonnes continuing the general stable situation of previous years. Aquaculture production amounted to 80.1 million tonnes in 2014, with 49.8 million tonnes of finfish, 16.1 million tonnes of molluscs, 6.9 million tonnes of crustaceans, and 7.3 million tons of other aquatic animals (FAO, 2016). World aquaculture production of fish accounted for 44.1% of total production (including for non-food uses) from capture fisheries and aquaculture in 2014. A total of 580 species and/or species groups were farmed around the world, including 362 finfishes, 104 molluscs, 62 crustaceans, 6 frogs and reptiles, 9 aquatic invertebrates, and 37 aquatic plants (FAO, 2016).

The apparent fish consumption per capita, increased from 9.9 kg in the 60´s to 19.7 kg in 2013. In 2013, fish accounted for 17% of global intake of animal protein and 6.7% of all protein consumed by world population (FAO, 2016). The growing demand of food fish by global population must be fulfilled by aquaculture production, in a way to allow overexploited fisheries stocks to be replenished. When compared to farm animals, fish is a rich source of high-quality proteins, micronutrients like phosphorus, iron and selenium, vitamins, and essential fatty acids (FAO, 2016; Tacon and Metian, 2013).

In Europe, according to Eurostat, aquaculture production reached 1.108 million tons and 3.365 billion euros in 2012 (STECF, 2014). The production value compared to 2011 increased 3.4% and, the production weight increased 3.8%. Four countries contribute to 70% of the total EU aquaculture value. These countries are United Kingdom (22%), France (21%), Greece (13%), and Spain (13%). In terms of total sales volume, the most produced marine species were Atlantic salmon (*Salmo salar*) (48%), gilthead seabream (*Sparus aurata*) (21%), and European seabass (*Dicentrarchus labrax*) (18%) (STECF, 2014).

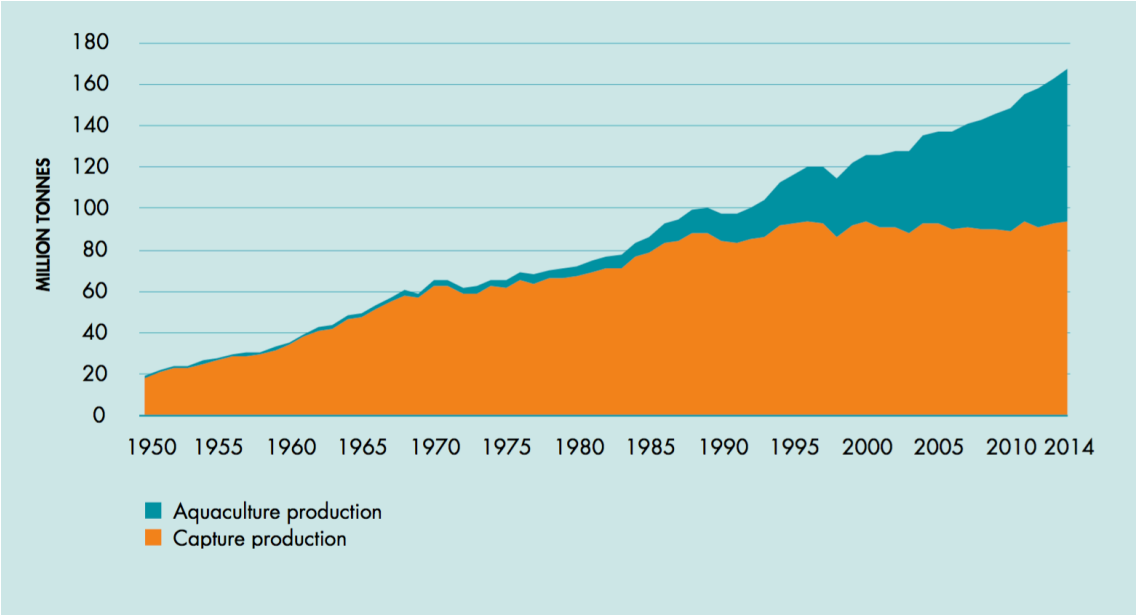

Fig. 1 - World capture fisheries and aquaculture production. Source: FAO (2016).

Portugal is considered the biggest fish consumer in Europe and the third largest consumer in the world (Almeida et al., 2015; Pieniak et al., 2013). In Portugal, the aquaculture sector produced around 10 thousand tonnes in 2014 and grossed 50.3 million euros (INE/DGRM, 2015). Compared to 2013 the production increased 7.2%. In contrast, the total value of production decreased 8.3% in the same period (INE/DGRM, 2015). This fact is explained by the increase production of turbot (*Scophthalmus maximus*) that decreased its market value. Production in marine and inland waters makes 93% of total aquaculture production. Of this value, fish production matches 47.7%. Of this, 91% of fish production is turbot and gilthead seabream (INE/DGRM, 2015). White seabream (*Diplodus sargus*) had 1 tonne produced in 2009 (FIGIS, 2016). Besides that, there are no reports of its production in Portugal.

Species diversification has been one of aquaculture´s developing strategies (Abellan and Basurco, 1999; Quemener et al., 2002). This strategy opens new markets, offering new species and thus, reducing the risk of fluctuating prices bringing the increase of the revenues to the industry. In the Mediterranean region, production became focused on high value species such as turbot, gilthead seabream and European seabass. White seabream arises as a new species with high interest. However, it still has a very limited production (Barazi-Yeroulanos, 2010).

1.2. White seabream (*Diplodus sargus*, Linnaeus, 1758)

White seabream belongs to the Sparidae family. This family includes 37 genera and 151 species. The individuals have an oval body with 5 black and 4 grey vertical bands (Fig. 2). The mouth is in the terminal position. The dorsal fin has 11 to 12 spines and 12 to 15 dorsal soft rays. The anal fin has 3 anal spines and 11 to 14 anal soft rays. The caudal fin is forked and the caudal peduncle has a dark saddle. Commonly the individuals are 25 cm long. The maximum length is 45 cm.

Fig. 2 - White seabream (Source: Rafaela Santos)

This species is abundant on the Atlantic shore from the Biscay bay to the Madagascar Island. They are also present in the Mediterranean Sea and rarely in the Black Sea. This species usually inhabits rocky bottoms from near the shore down to depths of 150 m (Morato et al., 2003). White seabream shows high site fidelity, but also have the ability to travel tens of kilometers during juvenile and adult phases (Abecasis et al., 2009; D'Anna et al., 2004; Di Franco et al., 2012; Di Lorenzo et al., 2014; Koeck et al., 2013).

They are omnivorous, preying gastropods, decapods, echinoderms, polychaetes, cnidarians and algae (Cejas et al., 2003; Figueiredo et al., 2005; Pallaoro et al., 2006; Sala and Ballesteros, 1997). They reproduce during the spring and the size at maturity is 16.7 cm (Morato et al., 2003).

White seabream has potential for Mediterranean aquaculture diversification because it has a high market value, reaching high prices. Farming trials began in the 1980´s with the description of the reproductive cycle, larval and embryonic development

(Coetzee, 1986; Divanach and Kentouri, 1982; Micale et al., 1987). The larval and post-larval stages have a high growth and survival rate (Cejas et al., 1993; Pousão-Ferreira et al., 1997). In latter stages the growth rate is slower (Abellan and Garcia-Alcazar, 1995). This could be specific of this species or it could be a consequence of the use of inadequate diets. In farming conditions they are mainly fed with commercial diets made for other species like gilthead seabream. Also, in rearing conditions, white seabream display a series of aggressive behaviours, which could also be related with the slow growth rates reported (Caballero and Castro-Hdez, 2003; Karakatsouli et al., 2007; Papoutsoglou et al., 2006).

White seabream juveniles have low protein requirements. Maximum growth performance is achieved with diets with 27% of protein content, while maximum protein retention requires a dietary protein level of 33% (Sa et al., 2008b). If the protein content is reduced to 15% the growth is depressed (Ozório et al., 2006). The low protein requirements are in agreement with the omnivorous feeding habits and with the slow growth rates shown by this species. Fast growing species have higher protein requirements than slow growers (Tacon and Cowey, 1985). Regarding the essential amino acid requirements, no data is available in white seabream juveniles.

Growth performance is not affected by diets with high fat content. Sa et al. (2008a) did not observed significant differences in the growth rate, diet utilization and whole-body composition with the increase of dietary lipids from 9% to 24% in 17 g fish. But, a trend for increasing whole-body lipid, energy content and visceral index with the increase of dietary lipids was observed. Lipids are mainly deposited in the viscera in white seabream (Ozório et al., 2006). Growth depression occurred when dietary lipid levels increased from 12% to 18% with fry weighting 1.5 g (Sa et al., 2006). No protein sparing effect due to dietary lipids occurs in regard to the existing data (Ozório et al., 2006; Sa et al., 2006; Sa et al. 2008a). No data regarding essential fatty acids requirements are available.

Waxy maize starch dietary inclusions of 36% are efficiently use by white seabream juveniles (Sa et al., 2007). Feeds can be formulated with a 38:36 protein: carbohydrate ratio without affecting the growth and feed utilization (FE). However, normal maize starch can be a more efficient energy source than waxy maize starch (Sa et al., 2008c). Dietary costs can be reduced with the low protein requirements and carbohydrates usage contributing to a decrease of N losses. This is a good

argument when considering the farming potential of this species. No data exists regarding vitamins and minerals requirements.

1.3. Fishmeal vs. Plant feedstuffs in aquafeeds

Aquaculture industry has been the largest consumer of fishmeal (FM) and fish oil (FO) that are the main protein and lipid sources in aquafeeds, respectively. Over the last 20 years with the growth of the aquaculture sector, the use of FM increased from 15% to 65% and the use of FO increased from 15% to 85% (Tacon and Metian, 2008).

Marine species are mostly carnivorous having high protein requirements ranging from 40% to 55% of the diet (NRC, 2011). In contrast, the majority of the freshwater species are omnivorous and herbivorous, having low protein requirements, ranging from 25% to 35% of diet. Marine fish require diets with high content of long-chain polyunsaturated fatty acids (LC-PUFA) namely 20:5n-3 and 22:6n-3, while freshwater species and most salmonids require C18-polyunsaturated fatty acids namely 18:3n-3 and 18:2n-6 (NRC, 2011).

FM has the ideal nutritional profile, resembling the nutritional requirements of most farmed aquatic species (NRC, 2011). FM has high protein content, an excellent amino acid profile, lack of anti-nutrients, high nutrient digestibility and high palatability. It is a rich source of minerals (like phosphorus), taurine, and vitamins (Hardy, 2010). FO have high digestibility and high content of essential fatty acids like LC-PUFA (Turchini et al., 2009).

However, due to the rising prices and the limited supplies, the aquaculture sector has reduced their reliance on FM and FO by using alternative protein and lipid sources (Bendiksen et al., 2011; Naylor et al., 2009; Turchini et al., 2009). In omnivorous fish, dependency on FM and FO is almost non-existent, and even in initial growth phases there is no apparent advantage of including animal protein in the diet (Sink et al., 2010). In carnivorous fish, reduction of FM and FO is more challenging (Tacon, 2004). Many aspects such price, protein content, amino acid profile, digestibility, essential amino acid deficiencies, anti-nutritional factors and palatability have to be considered to replace FM (Gatlin et al., 2007; Hardy, 2010).

Plant feedstuffs (PF) are the main alternative source to FM in aquafeeds (Tacon et al., 2011). PF have a high availability and a reasonable price. On the down side, PF have low protein content, several essential amino acids inadequacies and low palatability (Gatlin, 2007; Hardy, 2010). Another limitation is the presence of anti-nutritional factors like protease inhibitors, phytates, lectins, saponins, and non-starch polysaccharides (NSPs) (Francis et al., 2001). Some strategies were developed to overcome some of the disadvantages. Plant protein concentrates, supplementation of amino acids and feed attractants are used to surpass problems regarding low protein content, amino acid imbalances and low palatability (Davies et al., 1997; Dias et al., 1997; Watanabe et al., 2001). Although, some anti-nutritional factors are easily removed during processing, others are more difficult to remove. Addition of feed supplements can be used to overcome anti-nutritional factor issues (Francis et al., 2001).

1.4. Non-Starch Polysaccharides

PF nutritive value is limited by the presence of anti-nutritional factors, including high levels of NSPs (average 20-45% by weight) (Francis et al., 2001). NSPs refers to a large array of polysaccharide molecules, excluding *α*-glucans (starch), composed primarily by linked monomers of hexoses and pentoses like galactose, arabinose, glucose, mannose, and xylose (van Barneveld, 1999). NSPs can be divided in three groups: cellulose, non-cellulosic polymers and pectic polysaccharides. Based on the reaction with water, NSPs can be classified as soluble or insoluble. Cellulose is insoluble and other types of NSPs are soluble or partially soluble (e.g. guar gum, arabinoxylans, *β*-glucans, and mannans).

In fish, the enzymes needed to hydrolyse NSPs, like *β*-glucanases and *β*-xylanases, are rare or even absent (Kuz'mina, 1996). This way, NSPs are indigestible, thus remaining unavailable as energy source and becoming a source of organic pollutants. Moreover, NSPs may have detrimental effects on fish performance and health. NSPs viscous nature can interfere with gut epithelium, and mucus leading to alterations on digestive tract physiology and morphology (Sinha et al., 2011).
The viscous nature of soluble NSPs affects nutrient digestion and absorption leading to growth and FE depression (Amirkolaie et al., 2005; Leenhouwers et al., 2006; Leenhouwers et al., 2007a; Leenhouwers et al., 2007b; Refstie et al., 1999; Storebakken, 1985). Such an effect is possible related to the higher digesta

viscosity, which may bind nutrients and reduce interaction of digestive enzymes with subtracts. A reduction in nutrient digestibility due to an increase in digesta viscosity was observed in rainbow trout (*Onchorhynchus mykiss*) fed guar gum (Storebakken, 1985). In Atlantic salmon, dietary inclusion of soybean NSPs increased gut viscosity, affecting the digestion of amino acids and lipids (Refstie et al., 1999). In African catfish (*Clarias gariepinus*) inclusion of soluble NSPs increased digesta viscosity, affecting nutrient digestibility and gut fermentation activity (Leenhouwers et al., 2006; Leenhouwers et al., 2007a). Soluble NSPs can also bind the gut brush border increasing the thickness of the unstirred water layer adjacent to the mucosa, thus affecting nutrient absorption (De Lange et al., 2000). In African catfish, inclusion of cereal grains in the diet affected gastric emptying time, delaying gut absorption of glucose (Leenhouwers et al., 2007b).

Soluble NSPs may have impact on fish gut anatomy and development, being associated with increased size and length of digestive organs. In African catfish, an increase in the weight of digestive organs was observed in diets with high inclusions of guar gum (Leenhouwers et al., 2006). The gut morphology is also affected by dietary NSPs. Some enteritis-like changes can be found like the decrease or absence of absorptive vacuoles, infiltration of inflammatory cells in the lamina propria, and shortening of mucosal folding heights (Bakke-McKellep et al., 2000; Krogdahl et al., 2003; Krogdahl et al., 2010). In Atlantic salmon fed diets with NSPs from soy or cellulose, gut morphology alterations were found. In the distal intestine (DI) the changes included shortening and fusion of the simple mucosal folds, widening of the lamina propria with increased cellularity, leukocytic cellular infiltration of the submucosa and lamina propria, and reduced supranuclear vacuolization with apical nuclear displacement within enterocytes (Kraugerud et al., 2007).

The problems associated with the low digestibility of NSPs can be partially reversed by supplementing diets with NSPs-degrading enzymes. Exogenous enzymes such as xylanase and *β*-glucanase are extensively used as additives in farm animal diets to improve PF digestibility and decrease NSPs negative effects (Bedford and Cowieson, 2012). Supplementation of PF-based diets with carbohydrases as a strategy to improve NSPs utilization was also assessed in fish. Ai et al. (2007) fed Japanese seabass (*Lateolabrax japonicus*) with PF-based diets supplemented with NSPs-degrading enzymes (*β*-glucanase, pentosanase, cellulase, and xylanase). Fish fed diets with NSPs-degrading enzymes have a better growth performance

compared to the control group. Moreover, N retention was also enhanced with the use of NSPs-degrading enzymes. Lin et al. (2007) fed hybrid tilapia (*Oreochromis niloticus* x *O. aureus*) with diets with increasing levels of exogenous digestive enzymes (commercial enzyme complex of neutral protease, *β*-glucanase and xylanase). Specific growth rate, FE, protease and amylase activities significantly increased with the increasing of dietary enzyme levels. In contrast, there are studies reporting no effects of supplementation of PF-based diets with exogenous digestive enzymes. Ogunkoya et al. (2006) fed rainbow trout with PF-based diets supplemented with a commercial enzyme cocktail (xylanase, amylase, cellulase, protease and *β*-glucanase). Enzyme supplementation had no effect on fish growth, and on whole-body composition.

Overall, the efficacy of exogenous enzymes differs among fish species, due to the different enzymology parameters within their digestive systems (pH range, presence of cofactors, and enzyme resistance to proteolytic inactivation). Besides NSPs-degrading enzymes, gut bacteria characterized by a rich secretome have been recognized as potential sources of carbohydrases allowing the host to obtain energy from otherwise indigestible dietary constituents.

1.5. Functional ingredients - Probiotics

A functional ingredient may be or not a nutrient, and has a physiologic effect beyond the common nutritional effect, affecting one or more functions in the body, improving health or disease resistance (Roberfroid and Slavin, 2000). Probiotics (PRO) and prebiotics are functional ingredients and their incorporation in aquafeeds was considered a possible strategy to enhance PF utilization (Gatlin et al., 2007).

The word probiotic is constructed with the union of the Latin word pro, that means “for”, with the Greek word bios that means “life” (Zivković, 1998). There is not a standard definition of PRO. The first definition by Parker (1974) showed PRO as organisms and substances contributing to the gut microbiota balance. Fuller (1989) defined PRO as “...a live microbial feed supplement which beneficially affects the host animal by improving its microbial balance”. During the 90´s new definitions were proposed. Havenaar and Huis (1992) defined as a mono or mixed culture of live microorganisms to improve the properties of indigenous microflora. Skjermo and Vadstein (1999) defined as live gut bacteria that promote the viability of the host.

Lately, Merrifield et al. (2010b) summed up and applied the definition to aquaculture purposes. So, PRO is a live, dead or component of microbial cell administered via feed or rearing water benefiting the host by improving the disease resistance, health status, FE, growth performance, and stress response. This is achieved by improving the host's microbial balance or the microbial balance of the ambient environment.

To select a PRO, the following characteristics need to be satisfied (Merrifield et al., 2010b):

i) it cannot be pathogenic to host species but also with regards to aquatic animals and humans;
ii) it cannot be resistant to antibiotics;
iii) be resistant to bile salts and low pH;
iv) it must be able to adhere and grow in the gut mucus;
v) colonize the gut epithelial surface;
vi) be registered for use as a feed additive;
vii) it should display advantageous growth characteristics to the host;
viii) it should display antagonistic properties to one or more pathogens;
ix) it should produce extracellular digestive enzymes or vitamins;
x) it should be indigenous to the host or rearing environment;
xi) the PRO should remain viable in storage conditions and survive the industrial process.

Some of these requirements are essential (i, ii, iii), and the others are only favorable to the large-scale application.

Before using PRO in aquafeeds the following application methods must be considered:

- Type of probiont:

Under the Council Directive 70/524/EEC the list of PRO authorized in feeding stuffs are: *Bacillus cereus var. toyoi*, *Bacillus lichenformis*, *Bacillus subtilis*, *Enterococcus faecium*, *Lactobacillus casei*, *Lactobacillus farciminis*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Pediococcus acidilactici*, *Saccharomyces cerevisiae*, and *Streptococcus infantarius*. In fish, the most well documented bacteria used as PRO are *Bacillus* sp. and *Lactobacillus* sp. (Dimitroglou et al., 2011). Most commonly, PRO are administered singularly but various strains can be combined (Gatesoupe, 2002; Havenaar and Huis, 1992; Kesarcodi-Watson et al., 2008; Salinas et al., 2005). Besides these forms, PRO can be combined with prebiotics. This application is called synbiotic.

- Supplementation form:

Freeze-dried/lyophilised cells, dead cells, disrupted cells, cell-free supernatants, and spores are the most commonly PRO administration forms (Merrifield et al., 2010b). At an industrial level the use of spores, dead cells and lyophilised cells is more practical instead of culturing live cells. *Bacillus* sp. is one of the best bacterial species to use in PRO applications because of spore-forming abilities allowing a great viability after the pelleting process and have high resistance to gastric conditions (Casula and Cutting, 2002; Hong et al., 2005; Hyronimus et al., 2000).

- Dosage level:

PRO dosage is an important factor because different hosts have different responses to dietary PRO levels, bringing both positive and negative results (Bagheri et al., 2008; Nikoskelainen et al., 2001; Nikoskelainen et al., 2003; Panigrahi et al., 2004). Depending on the PRO species, host fish species, host physiological status, rearing conditions and the goal of feeding application, the appropriate level varies. The common PRO dosage is 10^8 to 10^9 colony forming units (CFU) g^{-1} feed when administered in the food and 10^5 CFU mL^{-1} when administered in the rearing water (Hai, 2015).

- Supplementation duration:

There are 3 distinct options to administrate PRO: a short-term application for the times of need; constant supplemented feeding and cyclic feed of supplemented diets (Merrifield et al., 2010b). Short-term supplementation of PRO has provided gastric colonization, protection against diseases, and stimulation of the immune system when administered before an infection (Brunt and Austin, 2005; Irianto and Austin, 2002a; Irianto and Austin, 2002b; Newaj‐Fyzul et al., 2007; Pieters et al., 2008). However, in a farming environment it is impossible to predict when a disease may occur, in order to provide PRO feeding in the weeks prior. Constant supply of PRO may provide benefits to fish, but there is little data available about this method of supplementation. Aubin et al. (2005) made a long-term application of PRO (during 5 months) and compared PRO recovery levels over time. It was observed that higher levels were obtained after 20 days of feeding (with *Pediococcus acidilactici* levels of log 2.5 CFU g^{-1} and *Saccharomyces cerevisiae* of log 4.5 CFU g^{-1}) when compared to 5 months of feeding (*Pediococcus acidilactici* levels of log 0.9 CFU g^{-1} and *Saccharomyces cerevisiae* were not detected). It is worth considering that the immune response is often diminished with the long-term use of immunostimulants

which leads to the immune status reverting back to control levels or, in extreme cases may lead to immuno-suppression (Bricknell and Dalmo, 2005; Sakai, 1999). Data are lacking considering the cyclic feed method. This strategy involves the PRO diets and unsupplemented diets being feed alternately for short periods (e.g. from 2 weeks to 4 weeks) cyclically. This method may provide protection against transient pathogens and also induce immune-stimulation (Balcazar et al., 2007; Nikoskelainen et al., 2003). This strategy helps avoiding the over-stimulation of the immune response observed with the long-term use of PRO.

1.5.1. PRO benefits and possible modes of action

PRO have many benefits to fish such as enhancing health status through improving immune system and disease resistance, improving growth performance, FE, carcass composition, digestive enzymes activity, gastric morphology, gastro intestinal (GI) colonization and thus microbial modulation, and water quality (Dimitroglou et al., 2011; Merrifield et al., 2010b) The modes of action that lead to the mention benefits are difficult to completely elucidate due to a wide range of possible modes of action and the synergistic relationships between them. Much of the knowledge available about the possible modes of action of PRO is derived from mammal's studies. Based on that, suggested modes of action of PRO in fish include colonization of the GI tract resulting in an increase of beneficial bacterial communities that promote the production of inhibitory compounds, competition for chemicals or available energy, competition for adhesion sites, inhibition of virulence gene expression or disruption of quorum sensing (Merrifield et al., 2010b).

1.5.1.1. Gut microbiota

The GI microbiota is the name given to the complex community and dynamic ecosystem that colonizes the GI tract of an animal. There are two major groups in the GI tract. The allochthonous microbiota which transits with the digesta, because they cannot colonize the mucus layer and the epithelial surface, and the autochthonous microbiota which is resident and can associate to the host tissues (Ringø and Birkbeck, 1999). Autochthonous microbiota can tolerate the pH of gastric juices and can resist the actions of bile acids giving the capacity to colonize the epithelial surface of stomach and gut (Savage, 1989). There are many functions of the GI microbiota in the host. They can promote nutrient supply, prevent the

colonization of infectious agents, maintain energy homeostasis, and normal mucosal immunity (Delzenne and Cani, 2008; Nicholson et al., 2005; Xu et al., 2003).

Data on the composition of gut microbiota in fish are controversial. To some authors most gut bacteria are aerobic or facultative anaerobic (Cahill, 1990). But there are data, in herbivore fish, where both facultative and obligate anaerobes are present in the gut (Clements, 1997). Some fish also have yeast colonizing their GI tract (Andlid et al., 1998; Gatesoupe, 2007). In marine fish, Gram-negative, facultative anaerobic bacteria such as *Acinetobacter*, *Alteromonas*, *Aeromonas*, *Bacteroides*, *Cytophaga*, *Flavobacterium*, *Micrococcus*, *Moraxella*, *Pseudomonas*, *Proteobacterium*, and *Vibrio* spp. are the most dominant bacteria genera of the GI tract (Romero et al., 2014). In contrast, the endogenous microbiota of freshwater fish species tends to be dominated by members of the genera *Aeromonas*, *Acinetobacter*, *Bacillus*, *Flavobacterium*, *Pseudomonas* representatives of the family *Enterobacteriaceae*, and obligate anaerobic bacteria of the genera *Bacteroides*, *Clostridium* and *Fusobacterium*. Various species of Lactic Acid Bacteria (LAB) (*Lactobacillus*, *Lactococcus*, *Streptococcus*, *Leuconostoc*, and *Carnobacterium* spp.) have been also demonstrated to comprise part of this microbiota (Romero et al., 2014).

Several factors affect the composition and establishment of GI microbiota in fish like fish developmental stage, the surrounding environment, rearing and farming conditions and diet composition (Nayak, 2010). Concerning diet composition, the replacement of FM by PF in fish diets led to changes in gut microbiota. In rainbow trout, the inclusion of 30% of soybean meal in the diets reduced the Proteobacteria and increased the Firmicutes (Desai et al., 2012). This work concludes that changes in gut microbiota structure can be partially responsible for the negative impacts of PF on fish growth and health. The microbiota of gilthead seabream GI tract was studied in fish fed diets comprising total replacement of FM (Estruch et al., 2015). Fish fed the vegetable mixture exhibited a higher percentage of Proteobacteria along the whole GI tract with the genus *Photobacterium* being one of the most represented. Hartviksen et al. (2014) studied the effect of the replacement of 45% of FM with various alternative dietary protein sources, namely soybean protein concentrate, pea protein concentrate and extracted sunflower in the gut microbiota of Atlantic salmon. When compared to the control diet, extracted sunflower had effects in the allochtonous microbiota with a significant increase in *Corynebacteriaceae* and *Lactobacillaceae* and a significant reduction in *β*-Proteobacteria, *Streptococcaceae* and *Peptostreptococcaceae*. Pea protein

concentrate resulted in a significant increase in *Vibrionaceae*, and soybean protein concentrate did not modulate allochthonous microbial community. In the autochthonous microbiota, the extracted sunflower inclusion decreased the levels of *Vibrionacea*, and pea protein concentrate caused a significant increase in *Enterobacteriaceae*. Soybean protein concentrate also decreased the levels of *Vibrionaceae*, but caused an increase in *Enterobacteriaceae*, *Lactobacillaceae* and *Streptococcaceae*.

Many studies assessed the impact of various PRO strains on the modulation of fish GI microbiota. Newaj-Fyzul et al. (2007) fed rainbow trout with a diet containing *Bacillus subtilis* at 10^7 CFU g^{-1} during 14 days. *Bacillus subtilis* was recovered at 5.3 x 10^4 CFU g^{-1} in the gut content and at 8.1 × 10^4 CFU g^{-1} in the gut mucus showing the establishment of this strain in fish gut. In Brown trout (*Salmo trutta*) inclusion of *Lactococcus lactis* spp. in the diet at 10^6 CFU g^{-1} was made during 2 weeks. Thereafter, non-supplemented feed was administered during 2 more weeks. Levels of PRO in the GI tract had a peak during the 2 weeks of feeding with the PRO diets and during the 2 weeks of non-supplemented, *Lactococcus lactis* spp. showed ability to persist in the gut (Balcazar et al., 2007). Zhou et al. (2012) studied the capacity of 10 *Lactobacillus* strains to adhere to the GI of zebra fish (*Danio rerio*). The strains were administered at 6.0 × 10^7 cells g^{-1} via feed during 4 weeks and led to elevated *Lactobacilli* levels in the GI tract (1.9 × 10^5 CFU g^{-1}) when compared to control (1.4 × 10^4 CFU g^{-1}). In general, PRO inclusions in the diets can modulate the GI microbiota of fish. However, there are some studies were such a modulation did not occur (Chang and Liu, 2002; He et al., 2011; Yang et al., 2012).

1.5.1.2. Growth performance

Some studies showed that PRO inclusion in the diets can enhance the growth performance of fish. Nile tilapia (*Oreochromis niloticus*) was fed during 9 weeks with a diet supplemented with 0.1% of a bacterial mixture containing *Streptococcus* sp. and *Lactobacillus acidophilus*. Results indicated that fish fed PRO supplemented diet exhibited greater growth than those fed with the control diet (Lara-Flores et al., 2003). In the same species, Wang et al. (2008) tested the inclusion of *Enterococcus faecium* at 1 x 10^7 CFU mL^{-1} in the rearing water during 40 days. Fish treated with PRO had an increase in growth performance when compared to the control group. In carnivorous species, studies also reported that PRO dietary supplementation can enhance fish growth. In European seabass larvae, a mix of *Lactobacillus farciminis*

and *Lactobacillus rhamnosus* administered in the diet showed benefits in growth, digestive enzymes activities as well as in the absence of malformations during fish development (Frouël et al., 2007). Suzer et al. (2008) fed gilthead seabream larvae with a mixture of *Lactobacillus* via live prey and rearing water. Fish submitted to the PRO treatment had a growth enhancement explained by the improvement of digestive enzyme activity. López et al. (2016) fed white seabass (*Atractoscion nobilis*) with diets supplemented with *Bacillus subtilis* at 1.9 x 10^7 cells g^{-1}. In supplemented diets, the growth performance increased alongside the feed intake, FE and protein and energy digestibility. Overall, the enhancement of growth performance due to dietary supplementation with PRO is related with the enhancement of digestive enzyme activity, improving digestion and thus feed utilization efficiency. In contrast, other studies showed no improvement in growth performance of fish fed with PRO (Merrifield et al., 2011; Shelby et al., 2007).

1.5.1.3. Gut morphology

The maintenance of a healthy gut microbiota is one of the keys to beneficially affect gut epithelial architecture (Merrifield et al., 2010b). The decrease of potential pathogens present within the GI tract may reduce mucosal damage and lead to enhanced absorptive surface area. Some studies have shown that PRO can improve gut morphology. Merrifield et al. (2010c) fed rainbow trout with *Bacillus* sp., and observed an improvement of microvilli length, which can lead to an enhancement in nutrient absorption.

Gut inflammation caused by PF-based diets can be decreased with dietary supplementation with PRO. Navarrete et al. (2013) fed Atlantic salmon with a soybean meal-based diet supplemented with LAB. Fish fed the PRO diet had the effects of soybean meal on lamina propria attenuated. Mucosal folds, goblet cells and supranuclear vacuoles improved somewhat with the addition of LAB. Some studies showed that the inclusion of PRO could increase the number of intraepithelial leukocytes (IEL) in the gut. Standen et al. (2015) fed Nile tilapia with a diet containing 37% of soybean meal and supplemented with a multi-species PRO (*Lactobacillus reuteri*, *Bacillus subtilis*, *Enterococcus faecium* and *Pediococcus acidilactici* (AquaStar® Growout)). Fish fed PRO supplemented diet had an increase of IEL populations when compared to the control group. Microvilli length and density was higher in PRO group. Similar results were observed in the same

species fed monospecies PRO applications of *Pediococcus acidilactici* (Standen et al., 2013).

1.5.1.4. Digestive enzymes

PRO effectively contribute to the digestive process of fish by producing extracellular enzymes such as proteases, carbohydrases and lipases (Ray et al., 2012). Various strains like *Bacillus* sp., *Enterobacteriaceae*, *Acinetobacter*, *Aeromonas*, *Flavobacterium*, *Photobacterium*, *Pseudomonas*, *Vibrio*, *Microbacterium*, *Micrococcus*, *Staphylococcus*, unidentified anaerobes, and yeasts were suggested to be exogenous digestive enzyme-producing organisms (Ray et al., 2012). Some enzyme-producing bacteria have been reported in fish gut. Khan et al. (2011) isolated phytase-producing strain CC 1.1 from Indian Carp (*Catla catla*) and identified it as *Rhodococcus* sp. German and Bittong (2009) reported β-xylosidase activity in the microbial extracts of three wood-eating catfish (*Panaque nocturnus, Hypostomus pyrineusi*, and *Panaque cf. nigrolineatus*) and one detritivores catfish (*Pterygoplichthy disjunctivus*). But the digestive activity was different between the gut fluids and microbial extract. The presence of any autochthonous xylanase-producing microbiota in fish gut requires further investigation. Microbial amylase, protease, lipase, tannase, and chitinase activities have also been documented in fish (Bairagi et al., 2002; Ray et al., 2012).

In fish, several studies reported an increase of digestive enzyme activity when fed diets with PRO inclusions. In grass carp (*Ctenopharyngodon idella*) fed diets with increasing levels of *Bacillus subtilis* (1×10^9; 3×10^9; 5×10^9 CFU kg^{-1}), an enhancement of protease, amylase and lipase activities in the gut were observed in all treatments when compared to the control group (Wu et al., 2012a). Askarian et al. (2011) fed beluga (*Huso huso*) and Persian sturgeon (*Acipenser persicus*) with *Lactococcus curvatus and Leuconostoc mesenteroides*. An increase of amylase, protease and lipase activities was observed. PRO positive effects on digestive enzymes activities have also been reported fish larvae (Suzer et al., 2008; Tovar-Ramirez et al., 2004). Yet, no data is available regarding the use of PRO strains capable of digesting NSPs.

1.5.2. Bacteria spores in animals feed

In nature, bacterial spores are a mean to survive extreme environmental conditions that could otherwise destroy vegetative bacteria (Nicholson et al., 2000). When the conditions are adverse, with the reduction in nutrients in the immediate vicinity of the live cell, the bacteria enters in a program of development resulting in the production of a spore in a time around 8 hours (Errington, 2003). The spore contains an endospore with a condensed and inactive chromosome. Surrounding the spore is a peptidoglycan-rich cortex and a coat made from layers of proteinaceous material (Henriques and Moran, 2007). This structure has a spherical or ellipsoidal shape with a length between 0.8 and 1.4 mm and is moderately hydrophobic. Spores are resistant to UV radiation, extreme heat (more than 80ºC), exposure to solvents, and enzymes like lysozyme (Nicholson et al., 2000). They have the ability to germinate if exposed to appropriate conditions. During the process, the water enters the spore and, the coat is removed along with outgrowth and restoration of vegetative cell growth (Moir, 2006). Spore-forming bacteria generally belong to one of two genera, *Bacillus* and the strictly anaerobic *Clostridia*, although many other genera also include spore-forming bacteria.

Heat-stable spores have several advantages for application in aquafeeds as spores are remarkably resistant to diverse insults including the harsh acid and bile salts gut conditions, are easily produced in large scale and can be dehydrated facilitating long term-storage and feed incorporation without losing characteristics (Barbosa et al., 2005; Spinosa et al., 2000). In aquaculture, bacteria spores are being extensively used as PRO for enhancing the growth and disease-resistance of cultured shrimps and in farm animal´s as growth enhancers and competitive exclusion agents (Cutting, 2011). However, in fish aquaculture only few data is available (Hong et al., 2005).

In a preliminary research project (PROFISH I - EXPL/MAR-BIO/0351/2013), it was isolated, identified and characterized more than 250 gut spore-forming bacteria (mainly *Bacillus* spp.) producing high levels of NSPs-hydrolysing enzymes. The observed carbohydrolytic profiles could be correlated with the presence of carbohydrate-active extracellular enzymes encoding genes on the isolates genomes. All isolates obtained were checked for minimal biosafety requirements to be eligible as PRO by the European Food Safety Authority. The adaptive fitness to the fish gut of selected isolates was inferred through different laboratory tests allowing to identify the best 4 candidates (FI92, FI99, FI142 and FI162) to become

PRO for improvement of fish health and utilization of dietary NSPs. Two of these isolates (FI99 and FI162) were further studied in the present master thesis.

2. Aims

This work aims at evaluating the most effective PRO to enhance the use of diets with a high content of PF in white seabream juveniles, without compromising fish health and welfare. For this, we used PRO previously isolated from European sea bass gut that were capable of digesting NSPs. PRO efficiency was evaluated through assessing its effects on:

- growth performance;
- feed utilization efficiency;
- the modulation of gut microbiota;
- digestive enzymes activities;
- gut integrity.

3. Materials and Methods

3.1. Spores production and purification

3.1.1. Production

Bacterial strains, stored at -80ºC, were grown in Luria Bertani (LB) agar plates (Becton, Dickinson and Company, USA) for 24h at 37ºC. Pure colonies were then used to induce sporulation by inoculating DSM broth (Difco Sporulation Medium) composed by Bacto Nutrient broth 8 g L^{-1}, 10% (w/v) KCl, 1.2% (w/v) $MgSO_4 \cdot 7H_2O$, 1 M NaOH, 1 M $Ca(NO_3)_2$, 0.01 M $MnCl_2$ and 1 mM $FeSO_4$ (pH 7.6). Sporulation occurred for 48h at 37ºC with agitation in an orbital shaker at 150 rpm.

3.1.2. Purification

Spore purification was done according to the method described by Tavares et al. (2013). The spores were centrifuged at 1900 g for 20 min and 4ºC. Cell pellets were suspendend in 50 mM Tris-HCl (pH 7.2) with 50 mg mL^{-1} of lysozyme and incubated at 37ºC during 1h. Then, cell pellets were washed with distilled water, and centrifuged before suspension in a 0.05% SDS (sodium dodecyl sulphate) solution, by vortexing. The samples were centrifuged and washed three times with distilled water, during three days, with two to three washes per day. At the end, samples were suspended in distilled water and frozen to be subsequently lyophilized and incorporated in the diets. Lyophilized spores were stored at room temperature and in the dark. The spore purity and yield was determined by plating serial dilutions in LB agar plates, before and after a heat treatment of 20 min at 80ºC to eliminate remaining (if any) vegetative cells.

3.2. Experimental diets

A control diet (CTR) was formulated to contain 48% of crude protein and 18% of crude lipid. FM and PF (soybean meal, rapeseed meal, sunflower meal, corn gluten and Dried Distillers Grains with Solubles) were used as protein sources at a ratio of 20:80 of protein from FM:PF, respectively. FO was used as the main lipid source. The other two diets were formulated identical with the incorporation of 2 lyophilised

pure spores preparations, FI99 and FI162, respectively at a dose commonly used in fish diets (2 x 10^9 spores g $feed^{-1}$). These 2 *Bacillus* sp. isolates with promising PRO characteristics were previously isolated from fish gut, selected, identified and characterized (Serra et al., 2014). Spores were added during the mixing of the ingredients. Ingredients composition and proximate analysis of the experimental diets is presented in Table 1. The ingredients were mixed and dry pelleted without steam in a laboratory pellet mill (California Pellet Mill, Crawfordsville, IN, USA) through a 3 mm die. Then, diets were dried in an oven at 40ºC for 24h and stored at 4ºC.

Table 1. Ingredient composition and proximate analysis of the experimental diets.

	Diets		
	CTR	FI99	FI162
Ingredients (% dry weight basis)			
Fish meal[a]	12.5	12.5	12.5
Soybean meal[b]	20.0	20.0	20.0
Rapeseed meal[c]	10.0	10.0	10.0
Sunflower meal[d]	10.0	10.0	10.0
Corn gluten[e]	24.5	24.5	24.5
DDGS[f]	3.4	3.4	3.4
Fish oil	14.4	14.4	14.4
Vitamin premix[g]	1.0	1.0	1.0
Mineral premix[h]	1.0	1.0	1.0
Choline chloride (50%)	0.5	0.5	0.5
Binder[i]	1.0	1.0	1.0
Bicalcium phosphate	1.7	1.7	1.7
Proximate analyses (% dry weight basis)			
Dry matter	95.3	94.6	95.4
Crude protein	48.8	48.9	48.6
Crude fat	18.3	18.3	18.1
Ash	6.9	7.0	6.8
Gross energy (kJ g^{-1} DM)	22.1	22.4	22.0

DM, dry matter; CP, crude protein; CL, crude lipid.

[a]Steam Dried LT, Pesquera Centinela, Chile. Sorgal, S.A. Ovar, Portugal (CP: 72.5% DM; CL: 11.7% DM).

[b]Sorgal, S.A. Ovar, Portugal (CP: 53.5% DM; CL: 2.0% DM).

[c]Sorgal, S.A. Ovar, Portugal (CP: 37.5% DM; CL: 4.0% DM).

[d]Sorgal, S.A. Ovar, Portugal (CP: 34.0% DM; CL: 1.0% DM).

[e]Sorgal, S.A. Ovar, Portugal (CP: 70.4% DM; CL: 3.7% DM).

[f]Dried Distillers Grains with Solubles: Sorgal, S.A. Ovar, Portugal (CP: 30.4% DM; CL: 11.8% DM).

[g]Vitamins (mg kg^{-1} diet): retinol, 18 000 (IU kg^{-1} diet); cholecalciferol, 2 000 (IU kg^{-1} diet); α- tocopherol, 35; menadione sodium bisulphate, 10; thiamine, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.

[h]Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dibasic calcium phosphate, 5.93 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.44 (g kg^{-1} diet).

[i]Aquacube. Agil, England (guar gum, polymethyl carbamide, manioc starch blend, hydrate calcium phosphate).

3.3. Fish and experimental conditions

White seabream juveniles were obtained from Instituto Português do Mar e da Atmosfera (IPMA), Olhão, Portugal. After transportation to the experimental facilities at the Marine Zoology Station (Porto University), fish were submitted to a quarantine period of 4 weeks. During that period fish were fed with a commercial diet (Skretting, Stavanger, Norway) containing 16% of lipid and 47% of protein. Thereafter, 9 groups of 12 fish with an initial body weight of 54.0 ± 0.1 g were established and each diet randomly assigned to triplicate tanks. Fish were fed by hand, twice a day, 6 days a week, until visual satiation. Utmost care was taken to assure that all feed supplied was consumed.

The experimental system consisted of a thermoregulated (23.0 ± 0.5ºC) recirculating seawater system (Fig. 3) equipped with a battery of fiberglass tanks with 100 L capacity. Tanks were supplied with continuous flow (2.5-3.5 L min^{-1}) of filtered seawater (35.0 ± 1.0 g L^{-1} salinity) and dissolved oxygen was kept near saturation (7 mg L^{-1}). Fish were subject to 12h light / 12h dark photoperiod regime provided by artificial illumination. The trial lasted 65 days.

Fig. 3 - Experimental system.

3.4. Sampling

Fish in each tank were bulk-weighed at the beginning and at the end of the trial, after 1 day of feed deprivation. For that purpose, fish were slightly anaesthetized with 0.3 ml L^{-1} ethylene glycol monophenyl ether (Sigma-Aldrich, Steinheim, Germany). At the end of the trial, 5 fish from each tank were randomly sampled 6h after the morning meal (to assure digesta in the gut) and euthanized with a sharp blow to the head. Gut with intestinal content was removed from 3 fish (Fig. 4), dissected on chilled trays. The digestive tract was freed from adjacent adipose and connective tissues. Circa 1 cm of the DI (distinguished from the mid gut by an enlarged diameter and darker mucosa) were sampled for histological evaluation. The samples were rinsed in phosphate buffered saline (PBS), carefully blotted dry with a paper towel, immediately fixed in phosphate buffered formalin (4%, pH 7.4) for 24h and subsequently transferred to ethanol (70%) until further processing. The remaining gut was immediately frozen in liquid nitrogen and then stored at -80ºC for the measurement of digestive enzymes activities. The 2 other fish were sampled under aseptic conditions for microbiota characterization. Digesta was collected by squeezing the entire gut. For mucosa sampling, the gut was open with sterile forceps and scissor in a sterile petri dish. A microscope slide was used to scrape the mucosa and collect it to sterile tubes. Both digesta and mucosa samples were immediately frozen in liquid nitrogen and stored at -80ºC.

Fig. 4 - Fish intestine excised (Source: Rafaela Santos).

3.5. Proximate analysis of the ingredients and diets

3.5.1. Crude Protein

Prior the analysis, diets were ground to obtain a homogenous sample. The protein content (Nitrogen (N) x 6.25) of ingredients and diets was determined using the Kjeldahl method, after acid digestion of samples, using a Kjeltec digestor and distillation units (Tecator Systems, Höganäs, Sweden, models 1015 e 1026, respectively) (Fig. 5). This method assumes that one protein contains 16% of N (100/16 = 6.25). Approximately 150 mg of each sample was weighed, in duplicate, into distillation tubes. Then, was added 1 Kjeldahl tablet containing selenium (Se), as a catalytic, plus 5 mL of sulfuric acid. Samples were digested 1h in the digestor unit at 450ºC. At the end of digestion, the organic N was converted in ammonium sulfate. After digestion and cooling, samples were then distilled in the distillation unit. Water and sodium hydroxide (NaOH, 40%) were added to each digestion tube and distillation was performed using saturated boric acid for ammonium sequestration. The final step consisted in quantifying N by titration with hydrochloric acid (HCl, 0.5 N), in the presence of a methyl orange pH indicator.

Fig. 5 - Kjeltec digestor and distillation units.

3.5.2. Crude lipids

The method used to determine the lipid content of ingredients and diets was the Soxtec method. The method consists of an extraction with petroleum ether in a Soxtec system (Tecator Systems, Höganäs, Sweden; extraction unit model 1043 and service unit model 1046) (Fig. 6). First, samples of 500 mg of ingredients and diets were put, in duplicates, in a cartridge and placed in the extraction unit. Then, 50 mL of petroleum ether was added to the extraction cups, previously identified and weighted, and positioned in the extraction unit. Samples were boiled for 1h in petroleum ether. Then, they were rinsed for 2h. The lipids were extracted to a cup. After extraction, the solvent was evaporated and the cup was dried in the oven. Lipids were estimated with the weight difference of the cups before and after the extraction.

Fig. 6 - Lipids extraction unit.

3.5.3. Moisture

The crucibles were weighted before placing 500 mg of ingredients or diet samples in them. They were put in an oven at 100ºC and dried until constant weight. The moisture content was determined through weight loss of samples plus crucibles.

3.5.4. Ash

The same crucibles, after the determination of moisture content, were put in a muffle furnace. They were incinerated at 450ºC for 16h and the inorganic residue obtained was weighted.

3.5.5. Gross energy

The gross energy content in a sample is defined by -ΔUc, which is the combustion energy at constant volume (kJ g^{-1}). Energy content of the diets was determined using an adiabatic bomb calorimeter (PARR Instruments, Moline, IL, USA; PARR model 6200). Approximately 200-500 mg of sample, depending of the predicted caloric value, was weighed, pelletized and combusted under a pressurized (2.53 x 106 Pa) oxygen atmosphere in the bomb. After combustion, the temperature in the 2 L water bucket surrounding the stainless steel bomb raised and it was measured and used to calculate the energy content in the sample. The apparatus was calibrated with benzoic acid, the conversion factor of 1 cal = 4.1814 Joule was applied.

3.6. Zootechnical parameters

Mortality, weight gain, daily growth index, feed intake, feed efficiency, protein efficiency ratio, N and lipid intake were calculated as follows:

- Mortality:

$$100\times\left(\frac{number\ of\ dead\ fish}{number\ of\ initial\ fish}\right)$$

- Weight gain (% initial weight):

$$100\times\left(\frac{final\ body\ weight-initial\ body\ weight}{initial\ body\ weight}\right)$$

- Daily growth index (DGI) (%):

$$100\times\left(\frac{final\ body\ weight^{1/3}-initial\ body\ weight^{1/3}}{time\ in\ days}\right)$$

- Feed intake (g kg average body weight^{-1}day^{-1}):

$$Dry\ feed\ intake\ \div\frac{\left(\frac{initial\ body\ weigth+final\ body\ weight}{2}\right)}{1000}\div time\ in\ days$$

- Feed efficiency (FE):

$$\frac{wet\ weight\ gain}{dry\ feed\ intake}$$

- Protein efficiency ratio (PER):

$$\frac{wet\ weight\ gain}{crude\ protein\ intake}$$

- N intake:

$$\frac{diet\ ingested \times \left(\frac{diet\ protein\%}{6.25}\right)}{\left(\frac{final\ weight + initial\ weight}{\frac{2}{1000}}\right)} \over days\ of\ trial$$

- Lipid intake:

$$\frac{diet\ ingested \times diet\ lipid\ content\ \%}{\left(\frac{final\ weight + initial\ weight}{\frac{2}{1000}}\right)} \over days\ of\ trial$$

3.7. Microbiota analysis

3.7.1. DNA extraction

DNA extraction was done according to Pitcher et al. (1989). Each sample was a pool of 2 fish of the same tank to reduce variability. Approximately, 300 mg of digesta and mucosa samples were weighted to a 2 mL bead-beater tube with 500 µL of STE buffer (0.1M NaCl, 10 mM Tris-HCl, 1mM EDTA, pH 8.0) and 0.5 g of glass beads (Sigma G8772). Samples were then homogenized twice for 30s in the bead-beater (BeadBug™, Benchmark Scientific, Edison, USA) at 2500 speed with an interval of at least 30s on ice. After 15 min of incubation at 75ºC, with gentle agitation every 5 min, tubes were centrifuged for 1 min at 13000 g and 500 µL of supernatant was transferred to new 2 mL tubes. The tubes after the addition of 100 µL of lysozyme (10 mg mL^{-1}) and 5 µL of RNAse (10 mg mL^{-1}) were incubated at 37ºC during 1h, followed by a 30 min incubation at 55ºC after being added 50 µL of 10% SDS and 3 µL of proteinase K (20 mg mL^{-1}). After 10 min on ice in the presence of 500 µL of GES solution (60 g guanidine thyicyanate, 20 mL EDTA 0.5 M, pH 8.0, 120 mL ddH_2O, 5 mL of 10% N-lauroylsarcosine solution) and 250 µL of 7.5 M ammonium acetate, a phenol-chloroform extraction was performed by adding 500 µL phenol-chloroform-isoamyl alcohol (25:24:1). Tubes were then centrifuged at 13000 g during 10 min. The upper aqueous phase was transferred to a new tube and 500 µL of chloroform:isoamyl-alcohol (24:1) was added. After another 10 min centrifugation at 13000 g, the aqueous phase was transferred to a new tube and

600 µL of isopropanol was added. After a 15 min incubation on ice and a 15 min centrifugation at 13000 g, the supernatant was carefully discarded and the DNA pellet was washed twice with 500 µL of 70% ethanol with a 10 min centrifugation at 13000 g after each wash. The DNA pellet was then dried at room temperature and ressuspended in 50 µL of ultrapure water, being then stored at 4ºC.

3.7.2. Polymerase Chain Reaction

Bacterial 16S rRNA gene fragments were amplified by a touchdown Polymerase Chain Reaction (PCR) on a T100^{TM} Thermal Cycler (Bio-Rad), using primers 16S-358F (which has a GC clamp at the 5´end) and 16S-517R (Muyzer et al., 1993), yielding a 233bp DNA fragment. PCR mixtures (50 µL) contained 24.75 µL of water (Sigma), 10 µL of GoTaq Buffer 5X (PROMEGA), 5 µL of each dNTPs (2 mM, PROMEGA), 2.5 µL of each primer (10 µM Forward and Reverse), 0.25 µL of GoTaq polymerase (PROMEGA), and 5 µL of DNA template. A 94ºC incubation for 5 min was followed by 10 cycles of 64ºC, 1 min, 65ºC, 1 min and 72ºC, 3 min. The annealing temperature was decreased at every cycle 1ºC, until reach 55ºC. Thus, final 20 cycles of 94ºC for 1 min, 55ºC for 1 min and 72ºC for 3 min. Final extension was at 72ºC, 10 min. The PCR products were resolved on a 1% agarose gel with GelRed (4µL/100mL) and 100mL of 1xTAE Buffer at 120V during 45min and visualized on a Gel Doc EZ System (Bio-Rad) with the Image Lab software v4.0.1 (Bio-Rad).

3.7.3. Polymorphism analyses of 16S rRNA genes by denaturing gradient gel electrophoresis (DGGE)

Electrophoresis occurred on a DCodeTM universal mutation detection system (Bio-Rad) and lasted 16h at 60ºC with 65V in a 1xTAE buffer. The gel was an 8% polyacrylamide gel with a denaturing gradient of 30 to 70% 7M urea/40%formamide. The gel was loaded with 10 µL of each PCR product. Gel was stained during 1h in 200 mL of 1XTAE buffer with 20 µL SYBR-Gold Nucleic Acid Gel Stain. The gel was imaged and photographed on a Gel Doc EZ System (Bio-Rad) with the Image Lab software v4.0.1. (Bio-Rad). Bands of interest were marked, excised and placed on Eppendorf tubes with 20 µL of ultrapure water and kept at 4ºC overnight to allow resuspension of the DNA. Tubes were then vortexed for 15s, centrifuged for 1 min at 13000 g and a subsequent PCR amplification was made similar to the one

described above but using a forward primer lacking the GCclamp. The amplified product was then sent to STABVIDA (Caparica, Portugal) for sequencing. The sequences were then analysed using the database BLAST (Basic Local Alignment Search Tool) at http://blast.ncbi.nlm.nih.gov/Blast.cgi.

3.8. Digestive enzymes activities

Intestines were homogenized (1:0.05 v/w) in ice-cold buffer (100 mM Tris-HCl, 0.1 mM EDTA and 0.1% (v/v) TritonX-100, pH 7.8). Homogenates were centrifuged at 33000 g during 30 min at 4ºC. The supernatants were collected and stored at -80ºC until analysis. The enzymes activities were measured in a microplate spectrophotometer reader (MultiskanTM GO, Thermo Scientific, Lisboa, Portugal).

3.8.1. α-Amylase activity

α-Amylase (E.C.3.2.1.1) activity was measured with a Spinreact kit (Girona, Spain, ref. 41201). The method comprises in the hydrolysis of 2-chloro-4-nitrophenyl-α-D-maltotrioside by α-amylase; this reaction releases 2-chloro-4-nitrophenol and forms 2-chloro-4-nitrophenyl-α-D-maltoside, maltotriose and glucose. The rate of 2-chloro-4-nitrophenol formation (molar extinction coefficient, 12.9 $mM^{-1}cm^{-1}$), is proportional to the catalytic concentration of α-amylase present in the sample. The reaction mix consisted of 200 µL of amylase reagent (2-chloro-4-nitrophenyl-α-D- maltotrioside) and 10 µL of diluted (1:1) sample homogenate. The reagent was incubated at 37ºC before being used. Absorbance (ΔDO/min) was read at 4s intervals during 3 min at 405 nm and 37°C.

3.8.2. Lipase activity

Lipase (EC 3.1.1.3) activity was measured using a Spinreact Kit (Girona, Spain, ref. 1001275). In this method, the pancreatic lipase along with the colipase, desoxycholate and calcium ions, hydrolyzes the substrate 1-2-O- dilauryl-rac-glycero-3-glutaric acid-(6'-methylresorufin)-ester. The rate of methylresorufin formation (molar extinction coefficient, 60.65 mM^{-1} cm^{-1}) is proportional to the concentration of catalytic lipase present in the sample homogenate. The reaction mix consisted in 200 µL of reagent 1 (40 mM TRIS pH 8.3, ≥ 1 mg L^{-1} colipase, 1.8

mM desoxycholate and 7.2 mM taurodesoxycholate), 40 µL of reagent 2 (15 mM tartrate pH 4.0, ≥ 0.7 mM lipase substrate and 0.1 mM calcium chloride (CaCl2) and 10 µL of sample homogenate. Reagent 1 was kept on 37ºC before being mixed in the reader plate. Absorbance (ΔDO/min) was then read at 10s intervals, during 12 min, at 580 nm and 37°C.

3.8.3. Total alkaline protease activity

Total alkaline protease activity was measured by casein-hydrolysis method according to Walter (1984) adapted by Hidalgo et al. (1999). A reaction mix containing casein (1% w/v 0.125 mL), buffer (0.1M Tris-HCl, pH 9.0; 0.125 mL) and homogenate supernatant (0.05 mL) was incubated at 37ºC during 1h. The addition of a 0.3 mL trichoroacetic acid (TCA) (8% w/v) solution stopped the reaction. The samples were then kept at 2ºC during 1h and centrifuged at 1800 g for 10 min. The supernatant absorbance was read at 280 nm against blanks. A control blank was made for each sample adding supernatant from the homogenates after incubation. To establish the calibration curve a tyrosine solution was used. Activity was measured based on the extinction coefficient for tyrosine (0.008 mL μg^{-1} cm^{-1}). One unit of enzyme activity was defined as the amount of enzyme needed to catalyze the formation of 1.0 µmol of tyrosine per min.

3.8.4. Trypsin activity

Trypsin activity was measured using Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride (1 mM BAPNA) as substrate and 50 mM Tris-base and 20 mM $CaCl_2$, pH 8.2 as buffer (Faulk et al., 2007). The reaction mix consisted in 10 µL of the diluted sample (1:9) and 70 µL of the solution previously prepared. Production of 4-nitroanaline (molar extinction coefficient, 8.8 mM^{-1} cm^{-1}) was monitored at 37ºC and followed at 410 nm (reads at 20s intervals during 15 min).

3.8.5. Specific enzyme activity

All enzyme activities were expressed as specific activity (mU mg^{-1} of soluble protein). Protein concentration was determined with Bradford´s method (1976) using

Sigma-Aldrich protein assay kit with bovine serum albumin solution as standard. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the hydrolysis of 1 µmol of substrate per min at assay temperature.

3.9. Histology processing and morphologic evaluation

DI samples were processed and sectioned using standard histological techniques. A small part of each sample was placed in an individual cassette and maintained in ethanol (70%) until processing. Samples were dehydrated with increasing ethanol concentrations (70%; 90%; 100%) with a carousel-type tissue processor (Citadel 2000 Tissue Processor, Thermo Fisher Scientific Inc, Massachusetts, USA) (Fig. 7), cleared with xylene and impregnated in paraffin. The samples were included in paraffin blocks in an embedding workstation (HistoStarTM Embedding Workstation Thermo, Fisher Scientific Inc, Massachusetts, USA) (Fig. 8).

Fig. 7 - Citadel 2000 Tissue Processor.

Fig. 8 - HistoStar™ Embedding Workstation.

Sections of 5 µm were cut from the block of paraffin with a microtome (Leica JungRM 2035) (Fig. 9). These sections were placed in a warm water bath before being collected to the glass microscope slide. The sections were stained with haematoxylin-eosin (H-E) in an automatic slide stainer (VaristainTM 24-4 Automatic Slide Stainer, Thermo Fisher Scientific Inc, Massachusetts, USA) (Fig. 10).

Fig. 9 - Microtome

Fig. 10 - Varistain™ 24-4 Automatic Slide Stainer.

Blinded evaluation was performed with particular attention to inflammatory changes, as previously described in salmon (Baeverfjord and Krogdahl, 1996; Krogdahl et al., 2003) namely: shortening of the mucosal folds, changes in enterocyte and supranuclear absorptive cells, connective tissue hyperplasia, and infiltration of inflammatory cells. A continuous score system from 1-5 was used as described by Penn (2011). In this scoring system score 1 was considered the normal tissue and score 5 the most altered tissue. The value of histomorphological alterations was calculated by averaging scores of the separate parameters. The images were acquired with the Zen software (Blue edition; Zeiss).

3.10. Statistical analysis

All statistical analyses were done using the SPSS 23.0 software package for Mac (IBM® SPSS® Statistics, New York, USA). Data are presented as means ± standard error of the mean (SEM). Statistical analysis of the zootechnical parameters and digestive enzyme activities was conducted using a one-way ANOVA. Data were tested for normality by the Shapiro-Wilk test and for homogeneity of variances by the Levene test. When normality was not verified values were transformed. The probability level for rejection of the null hypotheses was 0.05. Significant differences among means were determined using the Tukey multiple range test.

DGGE banding patterns were transformed into presence/absence matrices. The band intensities were measured using Quantity One 1-D Analysis Software v4.6.9 (Bio-Rad Laboratories, Lda. Amadora, Portugal). Relative similarities between

dietary treatments and replicates were calculated using Primer software v7.0.5 (PRIMER-E Ltd, Ivybridge, UK). Similarity percentages (SIMPER) were used to represent the relative similarities between treatments. Species richness was assessed using Margalef's measure of richness. Species diversity was assessed with the Shannon-Weaver index. Clustering of DGGE patterns was achieved by construction of dendrograms using the Unweighted Pair Groups Method with Arithmetic Averages (UPGMA). Microbial diversity parameters were subjected to a one-way ANOVA.

Histological data was neither normal nor homogeneous and could not be normalized, thus the Kruskal-Wallis non-parametric test and subsequent pairwise comparison was performed. The probability level of 0.05 was used for rejection of the null hypothesis.

4. Results

4.1. Growth performance and feed utilization efficiency

Data concerning growth performance and FE are described in Table 2. Final body weight, weight gain and daily growth index were similar among experimental treatments. Thus, and regardless the experimental diet, white seabream body weight increased from an initial value of 54 g to 73-74 g in 65 days. Feed, N and lipid intake were also not affected by PRO incorporation. Although not statistically significant, FE and protein efficiency ratio of fish fed the FI99 and FI162 diets were slightly higher compared to the control diet (p = 0.07 and 0.08, respectively). Mortality was low (< 6%) and not statistically significant.

Table 2. Growth performance and feed utilization efficiency of white seabream fed the experimental diets.

	Diets				
	CTR	FI99	FI162	*P* -value	SEM[1]
Final body weight (g)	72.7	74.2	74.2	0.70	1.28
Mortality (%)	5.6	5.6	0.0	0.27	2.27
Weight gain (% initial weight)	34.0	36.9	36.8	0.70	2.46
Daily growth index	0.59	0.63	0.63	0.69	0.04
Feed intake (g kg ABW^{-1}[§] day^{-1})	14.5	13.9	14.1	0.79	0.59
Feed efficiency	0.29	0.35	0.34	0.07	0.02
Protein efficiency ratio	0.58	0.71	0.69	0.08	0.03
N intake (g kg ABW^{-1}[§] day^{-1})	1.1	1.1	1.1	0.81	0.05
Lipid intake (g kg ABW^{-1}[§] day^{-1})	2.7	2.5	2.6	0.79	0.11

Mean values are presented for each parameter (n = 3). No significant differences were observed between dietary treatments (P > 0.05).

[1]Standar error of the mean (pooled).

[§]ABW: average body weight (initial body weight + final body weight)/2.

4.2. Microbial Diversity

The Bray-Curtis dendrogram and V3 16S rRNA DGGE fingerprints of bacterial communities (Fig. 11) indicate that both autochthonous (A) and allochthonous (B) population had similar profiles between dietary treatments, but the autochthonous population had more similar profiles (~75%) than the allochthonous one (~60%). In

both autochthonous and allochthonous microbiota dietary treatments CTR and FI162 have at least 2 out 3 replicates clustering together. In the autochthonous microbiota fish fed with FI162 diet seem to have a microbial community more closely related (above 95%) than fish fed with the CTR diet (under 90%) (Fig.11A). On the contrary, the allochthonous microbiota of fish fed CTR diet has a microbial community more related (above 85%) than fish fed FI162 diet (~80%) (Fig.11B).

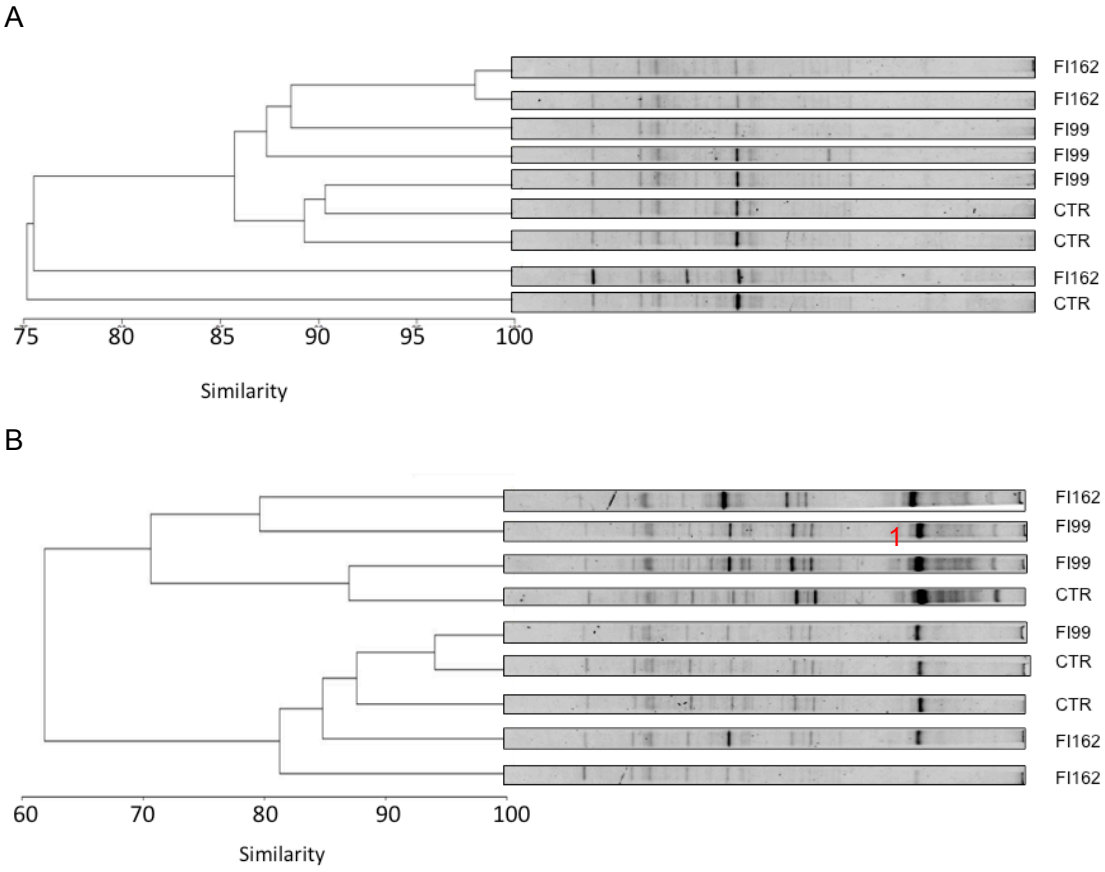

Fig. 11 - Dendrogram and PCR-DGGE fingerprints of the autochthonous (A) and allochthonous (B) gut microbiota of white seabream fed the experimental diets.

Sequence analysis from the DGGE band (band number 1 in Fig. 11) showed a 98% identity to *Lactobacillus aviarius* (Accession number NR_044703.2).

In both autochthonous and allochthonous gut microbiota the average number of OTUs, microbial richness, diversity, and similarity were not affected by dietary PRO supplementation (Fig. 12).

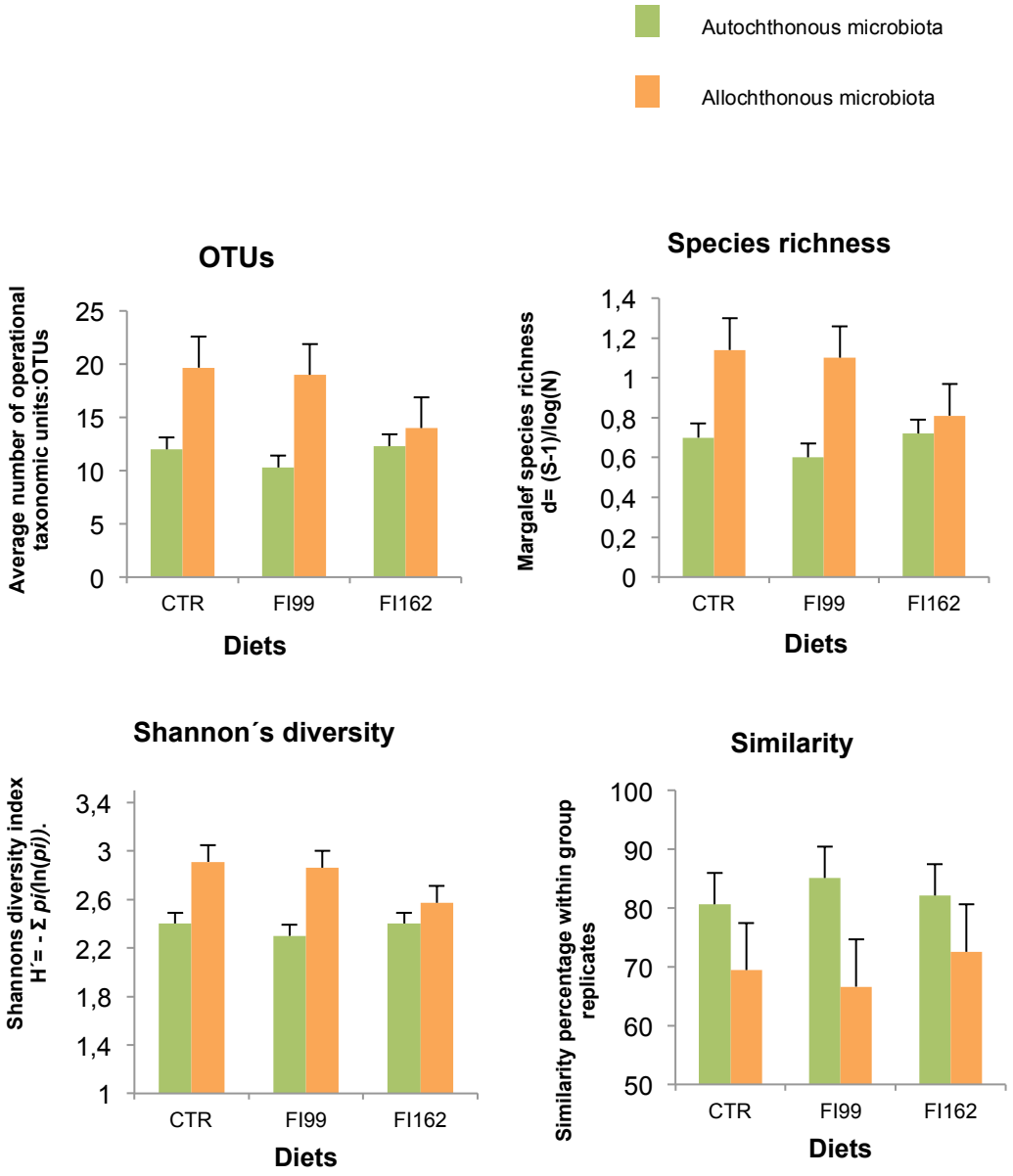

Fig. 12 - Ecological parameters obtained from PCR-DGGE fingerprints of autochthonous and allochthonous gut microbiota of white seabream fed the experimental diets. Values presented are means ± standard error of the mean (SEM) (n=3 per treatment pooled from six fish). No significant differences were observed between dietary treatments ($P > 0.05$).

4.3. Digestive enzymes activities

α-Amylase, lipase, total alkaline proteases and trypsin activities were unaffected by PRO incorporation (Table 3).

Table 3 - Specific activities (mU mg protein^{-1}) of digestive enzymes of white seabream fed the experimental diets.

	Diets				
	CTR	FI99	FI162	*P*-value	SEM[1]
α-Amylase	246.2	192.6	165.6	0.28	33.7
Lipase	2.01	1.56	1.82	0.47	0.25
Total alkaline proteases	238.8	220.5	248.7	0.59	19.5
Trypsin	124.1	73.7	73.8	0.34	23.2

Values presented as means (n=3). No significant differences were observed between dietary treatments (P > 0.05).

[1]Standard error of the mean (pooled).

4.4. Gut morphology

The DI of white seabream showed no signs of inflammation after the trial. The histological scores are statistically similar between all dietary groups (Table 4).

Table 4 - Details of score-based evaluation of gut histology (distal intestine) of white seabream fed the experimental diets.

	Diets				
	CTR	FI99	FI162	*P*-value	SEM[1]
Enterocyte nucleus position	1.7	1.6	1.4	0.11	0.29
Enterocyte vacuolization	1.0	1.0	1.0	1.00	0.00
Lamina propria width	1.4	1.4	1.3	0.85	0.45
Lamina propria leukocytes infiltration	1.2	1.0	1.0	0.10	0.20
Submucosa width	1.0	1.0	1.0	1.00	0.00
Submucosa leukocytes infiltration	1.0	1.0	1.0	1.00	0.00
EGC´s[2] infiltration	1.0	1.1	1.6	0.20	0.23
Mucosal folds height	1.0	1.0	1.0	1.00	0.00

A mean score was calculated from each of the above-mentioned characteristics. The values are means of the scores (n = 3). Results of Kruskal-Wallis all pairwise comparisons (P) are given.

[1]Standard error of the mean (pooled).

[2]Eosinophilic granular cell.

Some differences were found despite lacking statistical significance. In fish fed control diet (CTR) the nucleus position in the enterocytes was slightly misaligned when compared to other groups (Fig. 13). Enterocytes vacuolization (Fig. 14), mucosal folds height, and submucosa cellularity and width did not show differences between dietary treatments. The CTR diet showed some infiltration of leucocytes in lamina propria (Fig. 13). Fish fed both PRO containing diets presented some infiltration of eosinophilic granular cells in the submucosa and in the lamina propria (Fig. 15).

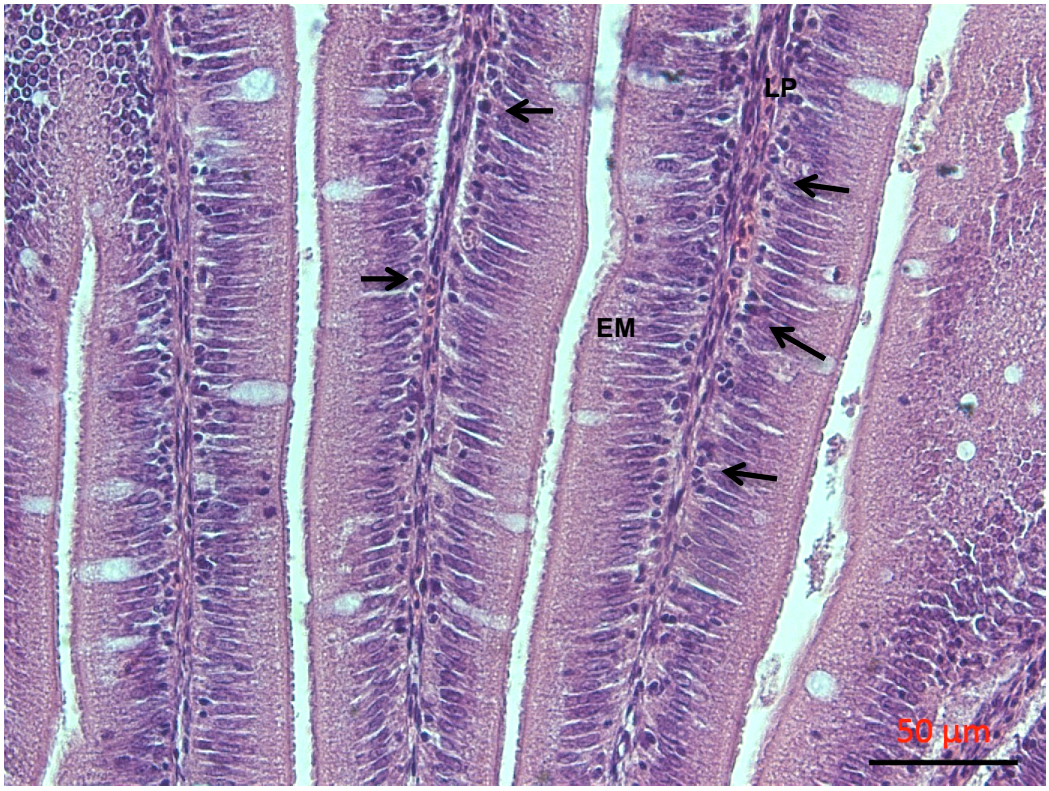

Fig. 13 - Distal intestine of white seabream fed control diet, depicting misalign enterocytes nucleous and leukocyte infiltration in the lamina propria. Black arrow: Intraepithelial leukocytes; EM: enterocytes slightly misaligned; LP: Lamina propria.

Fig. 14 - Details of enterocytes vacuolization and lamina propria in the distal intestine of white seabream fed FI99 diet. LP*: Lamina propria*; EN: Enterocyte nucleus aligned; V with black arrow: Enterocyte normal vacuolization.

Fig. 15 - Eosinophilic Granular Cells (EGC´s) infiltration in the distal intestine submucosa of white seabream fed FI162 diet. White arrows: EGC´s.

5. Discussion

The growth performance of white seabream observed in the present study confirms that juveniles have slow growth rates under culture conditions. Thus, in 65 days of trial, fish weight increased from an initial value of 54 g to a final value of 72-74 g in all treatments. Other studies with white seabream of similar body weight had growth rates within the same range, but slightly higher. Enes et al. (2015) fed white seabream of 55 g with a FM-based diet to apparent visual satiation 2, 3 or 4 times a day for 63 days. At the end of the trial, and independently of feed frequency, fish had a final weight of 88-90 g. White seabream of 41 g fed a FM-based diet with increasing levels of protein from 38 to 52% during 12 weeks, presented a final weigh ranging from 72 to 77 g (Sa et al., 2006). Overall, and in contrast to the mentioned studies, in our work white seabream were fed with a challenging PF-based diet, which may explain the slightly lower growth rates observed.

In aquaculture, an increasing interest has been rising regarding new dietary formulas with functional ingredients such as PRO. In fact, several studies performed in omnivorous, herbivorous and also in carnivorous species showed positive effects of PRO dietary inclusion in growth performance (Avella et al., 2010; Ayyat et al., 2014; Carnevali et al., 2006; Dawood et al., 2015; De Rodriganez et al., 2009; Frouël et al., 2007; Hamdan et al., 2016; López et al., 2016; Wu et al., 2012b; Xu et al., 2014). Growth improvements in fish fed diets with PRO inclusion were related to GI microbiota modulation, which enhanced digestive enzymatic activity due to bacterial digestive enzymes production, improving nutrients digestion, and thus feed utilization efficiency. However, present data contrast with the mention results since PRO did not improve growth performance of white seabream fed PF-based diets. An absence of PRO effect on fish growth was reported in fish fed diets supplemented with *Bacillus* sp. (Hidalgo et al., 2006; Merrifield et al., 2010a). Yet, no data are available on the effects of using *Bacillus* sp. spores, or spores from other bacterial strains, in fish diets.

FE and PER values were lower (0.29-0.35 for FE and 0.58-0.71 for PER) than the ones obtained in previous works (0.51-0.60 for FE and 1.26-1.42 for PER) with white seabream (Enes et al., 2015; Sa et al., 2006). Studies performed in other species of the *Diplodus* genus also reported higher FE and PER (0.59-0.67 for FE and 1.19-1.46 for PER) values for fish fed FM-based diets (Coutinho et al., 2016; Coutinho et al., 2014; Coutinho et al., 2012). As already mentioned for growth performance, the

high levels of PF inclusion in the diets may explain the lower FE and PER values obtained in the present study. Although not statistically significant, FE and protein efficiency ratio (PER) of fish fed the FI99 and FI162 diets were slightly higher compared to the CTR diet. An improvement of feed conversion ratio and PER was observed in fish fed PRO supplemented diets (De Rodriganez et al., 2009; Lopez et al., 2016; Merrifield et al., 2010a; Wu et al., 2012).

Modulation of gut microbiota due to dietary PRO supplementation has been reported in several fish species (Ferguson et al., 2010; Merrifield et al., 2010a; Ramos et al., 2013; Standen et al., 2015). However, in the present work, such an effect was not observed. A lack of gut microbiota modulation was also observed in common carp (*Cyprinus carpio*) fed diets supplemented with *Bacillus subtilis* for five weeks (He et al., 2011). Overall, the absence of PRO effect in gut microbiota modulation may explain the lack of a significantly PRO effect on growth performance and feed utilization efficiency.

PCR-DGGE is a very useful and inexpensive technique to assess microbial community and ecological characteristics, allowing the identification of bacteria that are not detected in culture-dependent methods (Zhou et al., 2014). As a semi-quantitative technique, PCR-DGGE may not be sensible to small changes in the bacterial community. So, it is possible that subtle changes in the abundance of OTUs may not be detected with this approach in the present study. This may explain the trend for a better FE in fish fed the PRO supplemented diets. In fact, it is possible that an undetectable microbiota modulation was responsible for a slight improvement of nutrient digestibility, and thus of FE. Quantitative methods such as FISH, qPCR and next generation sequencing, as well as the performance of digestibility studies, and the assessment of nutrient digestibility, namely NSPs are further required.

Comparing PCR-DGGE fingerprints of autochthonous and allochthonous microbiota microbiota, one band appears exclusively in the latter and it was identified as *Lactobacillus aviarus*, a genus of the LAB clade. This strain was first isolated in the GI tract of broiler chickens and although not dominant, LAB bacteria are often found in the gut of several fish species (Fujisawa et al., 1984; Ringo and Gatesoupe, 1998). *Lactobacillus aviarus* was also identified in allochthonous microbiota of gilthead seabream and turbot fed PF-based diets supplemented with the prebiotic short-chain fructooligosaccharides (Guerreiro et al., 2016a; Guerreiro et al., 2016b).

The digestive enzyme pattern usually reflects fish feeding habits. In fact, Castro et al. (2013) recorded higher amylase activity in white seabream gut compared to meagre (*Argyrosomus regius*), which is a carnivorous species. Accordingly, in the present study, a high amylase activity was recorded in all treatments. This reflects white seabream omnivorous habits and supports the high capability of this species to tolerate high carbohydrate levels in the diet. Thus, Sa et al. (2008c) reported that white seabream growth performance was unaffected with dietary carbohydrate levels up to 42%. α-Amylase activities recorded in the present study were higher (166-246 mU mg protein^{-1}) than the ones (43-61 mU mg protein^{-1}) obtained by Castro et al. (2013) in the same species using a similar enzymatic assay. Although, not measure in the present study, it is possible that our diets have a higher starch content compared to the ones of Castro et al. (2013).

Proteolytic activity was higher in white seabream. This is in agreement with the high efficiency for dealing with dietary protein that is generally observed in fish (Cowey, 1995). Indeed, unlike amylolytic activity, protease activity seems to be less dependent on fish nutritional habits (Chan et al., 2004; Hidalgo et al., 1999). Higher protease activities (221-249 mU mg protein^{-1}) were recorded in the present study compared to the work (88-135 mU mg protein^{-1}) of Castro et al. (2013). Since dietary protein content was similar in both studies, a possible explanation for the higher protease, and also amylase activities, recorded in our study may be differences in gut digesta levels. Thus, our samples were obtained 6h after the last meal, whereas no reference to gut sampling time was made on the work of Castro et al. (2013).

PRO effectively contribute to the digestive process of fish by producing extracellular enzymes such as proteases, carbohydrases and lipases (Bairagi et al., 2002; Lazado et al., 2012; Ray et al., 2012). In white seabream, dietary inclusion of brewers spent yeast (*Saccharomyces pastorianus*) at 1 or 2% enhanced α-amylase and protease activities in the gut (Castro et al., 2013). However, in the present work, PRO failed in enhancing the activity of the digestive enzymes assessed. This is in agreement with the lack of PRO effect in modulating white seabream gut microbiota. Similar results were recorded in other studies (Koca et al., 2015; Sun et al., 2012; Sun et al., 2011). Further studies are needed, namely the assessment of the activity of NSPs degrading enzymes, to truly understand PRO effect on host digestive process.

PF based-diets, namely soybean containing diets, can cause enteritis-like changes such as decrease or absence of absorptive vacuoles, shortening mucosal folding

heights, and profound infiltration of lamina propria inflammatory cells (Baeverfjord and Krogdahl, 1996; Krogdahl et al., 2000; van den Ingh et al., 1991). These effects are related with the anti-nutrients, namely NSPs, presented in PF and are particularly important in salmonids, while in species such as the European seabass and gilthead seabream such negative effects are less evident (Couto et al., 2015; Couto et al., 2014). In the present study, DI of white seabream showed no explicit signs of inflammation after the trial. Omnivorous species have higher capacity to adapt to PF in the diets, when compared to carnivorous fish. During 5 weeks, Uran et al. (2008) fed common carp with diets in which FM was partially replaced by 20% of soybean meal. DI showed signs of enteritis in the first 2 weeks of feeding that were faded during the other half of the study. No morphological alterations were found in the DI of white seabream fed diets with 12% of guar gum, a soluble NSP (Enes et al., 2012).

PRO seems to be effective in modulation gut morphology by altering the width, height, muscle layer thickness, surface area of the villi, thus improving GI tract absorption capacity (Liu et al., 2007). Positive effects of PRO in fish gut morphology of fish were reported in both carnivorous and omnivorous species (Merrifield et al., 2010a; Navarrete et al., 2013; Pirarat et al., 2011; Romarheim et al., 2011; Standen et al., 2015; Standen et al., 2013). In the present study, PRO inclusion in the diets had minor effects in white seabream gut morphology. Similarly, an absence of *Bacillus* spp. or *Enterococcus faecium* effect on microvilli morphology was reported in rainbow trout (Merrifield et al., 2010c). As the positive effects of PRO on gut morphology are due to a gut microbiota modulation, the lack of significant PRO effect in gut morphology, observed in our study, is in agreement with the lack of modulation of the gut microbiota.

6. Conclusions

The results of the present thesis allowed the following conclusions:

- Growth performance of white seabream confirms that juveniles have slow growth rates under culture conditions.
- PRO dietary supplementation had no remarkable effect in overall fish performance.
- Although not statistically significant, FE and PER of fish fed PRO supplemented diets were slightly higher compared to the CTR diet.
- No PRO effect in gut microbiota communities were detected by PCR-DGGE.
- PRO failed in enhancing α-amylase, lipase, proteases and trypsin activities.
- No explicit signs of inflammation were observed in DI, showing that omnivorous species have higher capacity to adapt to PF-based diets.
- PRO inclusion in the diets had minor effects in white seabream gut morphology.

Overall, dietary PRO supplementation failed in modulating both autochthonous and allochthonous gut microbiota which may explain the lack of PRO effect on growth performance, feed utilization efficiency, digestive enzymes activities and gut morphology. Thus, based on the parameters assessed and considering cost-benefit, does not seem worthy of including these PRO in white seabream juveniles PF-based diets.

One of the main conclusions of this thesis is that PRO lack in modulating gut microbiota community. Future works should address if *Bacillus* sp. spores indeed germinate in fish GI tract. Quantitative methods such as FISH, qPCR and next generation sequencing, should be used to assess gut microbiota community, in order to overcome PCR-DGGE limitations.

The trend for the enhancement of FE and PER with dietary PRO inclusion needs to be further studied. Thus, a digestibility trial should be performed. Besides α-amylase, future studies should address the activity of other carbohydrases, namely NSPases. Due to the slow growth of juveniles of this species in culture conditions, a longer growth trial (about 3 months) should also be performed to confirm the mentioned trends.

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